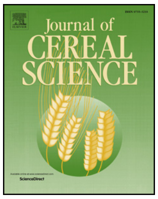
Journal of Cereal Science 85 (2019) 206–213

Contents lists available at ScienceDirect

Journal of Cereal Science

journal homepage: www.elsevier.com/locate/jcs



# Identification of low phytic acid and high Zn bioavailable rice (*Oryza sativa* L.) from 69 accessions of the world rice core collection

Ishara Perera[a,b], Ayaka Fukushima[c], Mako Arai[a], Kenta Yamada[c], Seiji Nagasaka[a,c], Saman Seneweera[d,e], Naoki Hirotsu[a,c,d,*]

[a] *Graduate School of Life Sciences, Toyo University, 1-1-1 Izumino, Itakura-machi, Oura-gun, Gunma 374-0193, Japan*
[b] *Grain Legumes and Oil Crops Research and Development Centre, Department of Agriculture, Angunakolapelessa, Sri Lanka*
[c] *Faculty of Life Sciences, Toyo University, 1-1-1 Izumino, Itakura-machi, Oura-gun, Gunma 374-0193, Japan*
[d] *Centre for Crop Health, University of Southern Queensland, Toowoomba, QLD 4350, Australia*
[e] *National Institute of Fundamental Studies, Hantana Road, Kandy, Sri Lanka*

ARTICLE INFO

*Keywords:*
Bioavailability
Genome-wide association study
Phytic acid
Zinc

ABSTRACT

Improving the micronutrient content and reducing the phytic acid (PA) in major staple food crops through plant breeding techniques are considered sustainable strategies to increase micronutrient bioavailability. This study documents the variation in PA and zinc (Zn) contents within the natural genetic variation of rice using the World Rice Core collection (WRC) and identifies useful genetic determinants in the Zn biofortification process. Some WRC accessions were observed having a low PA content and high Zn bioavailability and some others with a high PA content and low calculated Zn absorption. No significant differences were observed in the mineral or heavy metal contents among low and high PA lines examined suggesting that different mechanisms are controlling these traits, so that manipulating the PA content could be achieved without affecting the concentration of these elements. A genome-wide association study revealed that a chromosomal region near several significant SNPs determines the natural variation in rice PA content. Furthermore, a low PA trait in rice, rather than a high Zn content should be the key target for increasing Zn bioavailability.

## 1. Introduction

Micronutrients are essential elements for the effective functioning of human metabolic activities and good health. Currently, micronutrient malnutrition or hidden hunger is a global health issue caused by inadequate intake of essential vitamins and minerals from the diet. Zinc (Zn) is an essential micronutrient required for proper growth and development, immune system function, reproductive health and neurobehavioral development in the human body (Brown et al., 2004). Zn deficiency causes impaired growth, increased susceptibility to infections and increased mortality and affects around one-third of the world's population (WHO, 2002). Populations in developing countries are at a high risk of Zn deficiency due to high intake of plant-based diets (IFPRI, 2016). Among the strategies to overcome Zn deficiency, Zn biofortification is considered to be a major solution that appears to be the most sustainable and cost-effective approach for addressing this global nutritional issue (Miller and Welch, 2013).

Rice (*Oryza sativa* L.) is one of the world's most important crops, providing nutrition for approximately one-half of the global population and is the most important crop in Asia (FAO, 2013). Compared to other cereals, rice is a poor source of essential micronutrients to fulfill daily human nutritional requirements. Therefore, even a slight improvement in the nutrient content would benefit a large population around the world, especially in developing countries. Accordingly, many kinds of research have been carried out to develop Zn-biofortified rice during the past decade to achieve a target level of 28 μg Zn/g polished rice as specified by the HarvestPlus program or approximately 30% of the estimated daily average requirement (Trijatmiko et al., 2016). On the other hand, antinutrients present in cereals such as phytic acid (PA) substantially reduce the absorption of Zn inside the human intestine (Al Hasan et al., 2016).

Phytic acid (*myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate) is the principle storage form of phosphorus (P) in cereals and legumes and accounts for 65%–85% of the total seed P (Raboy, 2001). PA has six negatively charged ions, making it an effective chelator of many essential micronutrients, such as magnesium (Mg), calcium (Ca), iron (Fe) and zinc (Zn). The resulting phytate-mineral salts reduce the absorption of mineral nutrients in humans and non-ruminant animals. PA intake is

---

[*] Corresponding author. Faculty of Life Sciences, Toyo University, 1-1-1 Izumino, Itakura-machi, Oura-gun, Gunma 374-0193, Japan.
*E-mail address:* hirotsu@toyo.jp (N. Hirotsu).

https://doi.org/10.1016/j.jcs.2018.12.010
Received 22 October 2018; Received in revised form 13 December 2018; Accepted 14 December 2018

high in developing countries where people depend mainly on staple foods such as rice which then increases the risk of Zn deficiency (Lott et al., 2000). Accordingly, micronutrient-enriched cereals with a low PA content are considered to be a promising approach for increasing the bioavailability of minerals in humans (Welch and Graham, 2004).

As an effective approach for minimizing the adverse effects of PA in cereals and, thereby improving micronutrient bioavailability, mutation breeding has been successfully used to generate low phytic acid (*lpa*) crops through γ-irradiation or chemically induced mutagenesis (Liu et al., 2007; Lott et al., 2000). Several genes responsible for PA accumulation in rice grain have been identified, including eight genes that are expressed in developing rice grains (Perera et al., 2018; Sato et al., 2013).

To date, there are some reports assessing the natural variation in the PA content of rice (Lee et al., 2014; Liu et al., 2007; Wang et al., 2011). Thus, efforts to improve micronutrient bioavailability in rice has seemed challenging until the present. Proper exploitation of the variation existing within the rice germplasm should be beneficial toward improving rice Zn bioavailability through conventional or marker-assisted breeding programs. Genome-wide association study (GWAS) is an efficient tool that has been widely used to identify the genetic variation of complex traits in plants (Zhao et al., 2011). GWA mapping has been successfully used to identify numerous Quantitative Trait Loci (QTL) for agronomically important traits and also to assess variations in grain elemental compositions in rice (Spindel et al., 2015; Xu et al., 2016). So far, only two QTL have been identified in rice for the PA content (Stangoulis et al., 2007).

This study was aimed at identifying low PA rice from the natural variation of rice and understanding the genetic basis of natural variation in PA content in the WRC. In this study, we first identified the natural variation of PA and Zn contents in rice and evaluated the impact of PA on Zn bioavailability. Further, the micronutrient and heavy metal contents of accessions identified as low and high PA rice were determined to document effects of the PA content on other elemental contents owing to their importance in human nutrition and health. Finally, a GWAS was conducted to identify the significant genetic polymorphisms controlling PA content in the world rice core collection (WRC).

## 2. Experimental

### 2.1. Plant materials

The study was conducted using 69 accessions of the World Rice Core collection (WRC) (Kojima et al., 2005) obtained from the National Institute of Agrobiological Sciences (NIAS) Genebank in Tsukuba, Japan (Genebank Project, NARO). These accessions represent a wide geographical location worldwide. First, all the 69 WRC accessions were transplanted at the experimental field in Itakura, Gunma, Japan in May 2017. Among them, the accessions which the panicles did not emerge (WRC 58, WRC 59, WRC 60, WRC 61, WRC 62, WRC 63, WRC 65, WRC 66, WRC 67, WRC 68 and WRC 97) even after one month of other accessions, were transferred to pots and maintained in the glasshouse until harvest. Harvested seeds were dried to 13% moisture and stored at room temperature for further experiments.

### 2.2. Determination of PA content

The PA content of brown rice samples in the WRC was determined using a Phytic Acid Assay Kit (Megazyme International, Ireland) with minor modifications to the protocol (McKie and McCleary, 2016). Biological sample of one crushed grain was used with three replicates (n = 3) for the analysis. The crushed rice grain was digested with 500 μl of HCl (0.66 M) placed in a mixer overnight at room temperature. Aliquots (200 μl) of the extract were transferred to Eppendorf tubes and centrifuged at 3000 *g* for 20 min. Then, 100 μl of the supernatant liquid was neutralized by adding 100 μl 0.75 M NaOH in a new tube. For the free phosphorus determination, 12.5 μl of sample extract was mixed with 155 μl distilled water and 50 μl phytase assay buffer and incubated at 40 °C for 1 h. Distilled water (5 μl) and 50 μl of Alkaline phosphatase (ALP) assay buffer were added and the samples were incubated at 40 °C for 1 h. For the total phosphorus determination, 12.5 μl sample extract was mixed with 150 μl distilled water, 50 μl phytase assay buffer and 5 μl of phytase and samples were incubated at 40 °C for 1 h. ALP assay buffer (50 μl) and 5 μl of ALP were added to samples, followed by a 1 h incubation at 40 °C. For both the free phosphorus and total phosphorus determinations, 75 μl of 50% (w/v) trichloroacetic acid was added to all tubes to terminate the reactions, followed by centrifugation at 3000*g* for 15 min. Colour reagent (50 μl) was added to 100 μl of the supernatant liquid, and samples were incubated at 40 °C for 1 h. A phosphorus calibration curve was prepared according to the manufacturer's protocol. The absorbance at 655 nm for each sample and standard was recorded, and the phosphorus and phytic acid contents were calculated following the manufacturer's instructions.

### 2.3. Determination of mineral, heavy metal and Zn contents

Dehusked rice seeds of 69 WRC accessions were digested with concentrated $HNO_3$ in Teflon cases heated on a hotplate starting at 80 °C and increasing up to 150 °C at 30 min intervals. The Zn content of the digested samples was analyzed by inductively coupled plasma mass spectrometry (ICPMS, XSERIES 2, Thermo scientific, MA, USA) according to the manufacturer's instructions. Each accession was analyzed in triplicate.

Accessions identified as low PA (WRC 5, WRC 12 and WRC 30) and high PA (WRC 6, WRC 22 and WRC 44) WRC were analyzed for calcium (Ca), manganese (Mn), iron (Fe), and copper (Cu) contents. Heavy metals, arsenic (As), cadmium (Cd), tin (Sn), mercury (Hg), and lead (Pb), were also analyzed by the same ICPMS procedure.

### 2.4. Calculation of Zn bioavailability in rice

Using the measured PA and Zn contents, the Zn bioavailability in the WRC accessions was calculated from a mathematical equation developed by Miller et al. (2007) as follows:

$$TAZ = 0.5\left(A_{MAX} + TDZ + K_R\left(1 + \frac{TDP}{K_p}\right) - \sqrt{\left(A_{MAX} + TDZ + K_R\left(1 + \frac{TDP}{K_p}\right)\right)^2 - 4{\cdot}A_{MAX} + TDZ}\right)$$

where TAZ, the total daily absorbed Zn (mg Zn/day); $A_{\mathrm{MAX}}$, maximum absorption; $K_{\mathrm{P}}$, the equilibrium dissociation constant of the Zn-phytate binding reaction; $K_{\mathrm{R}}$, the equilibrium dissociation constant of the Zn-receptor binding reaction; TDP, the total daily dietary phytate (mmol phytate/day); and TDZ, the total daily dietary Zn (mmol Zn/day). The parameters, $A_{\mathrm{MAX}}$, $K_{\mathrm{R}}$, and $K_{\mathrm{P}}$ values were set as 0.091, 0.680 and 0.033, respectively as found for Zn homeostasis in the human intestine (Hambidge et al., 2010) The model predicts TAZ based on the total daily dietary PA intake (TDP) (mmol PA/day) and total daily dietary Zn intake (TDZ) (mmol Zn/day). The average daily intake of rice per person was defined as 400 g/day, either as brown rice or milled rice (Mottaleb and Mishra, 2016) and the TAZ was calculated based on the reference 400 g of rice per day.

### 2.5. Correlation among phenotypic traits of WRC

Data on phenotypic traits, including the number of days to heading, culm length, panicle number, panicle length, grain length, grain width, and amylose content, were obtained from the data available at the

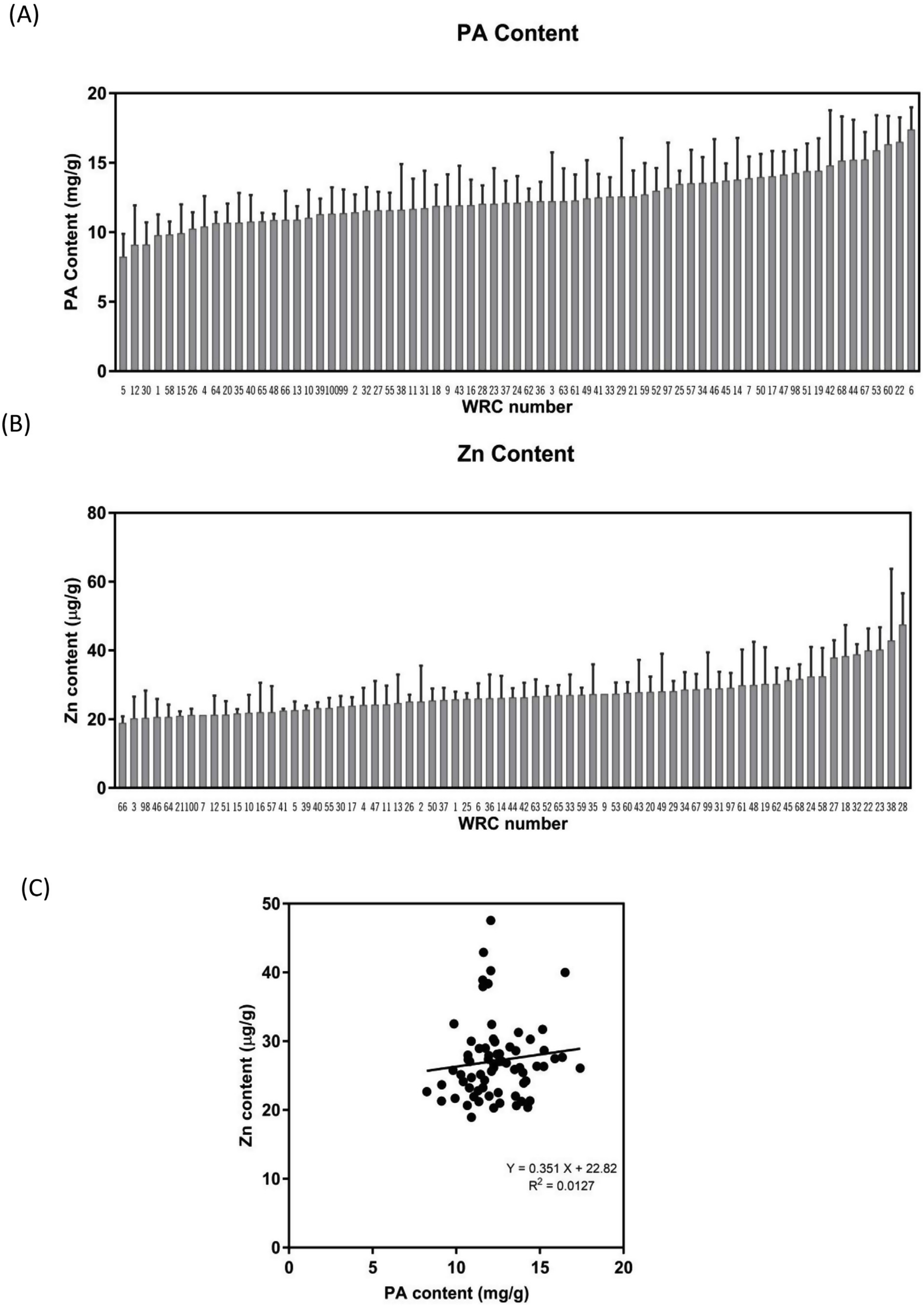


**Fig. 1.** Evaluation of 69 brown rice accessions in the WRC for (A) PA content (B) Zn content (C) Correlation between the PA and Zn contents.

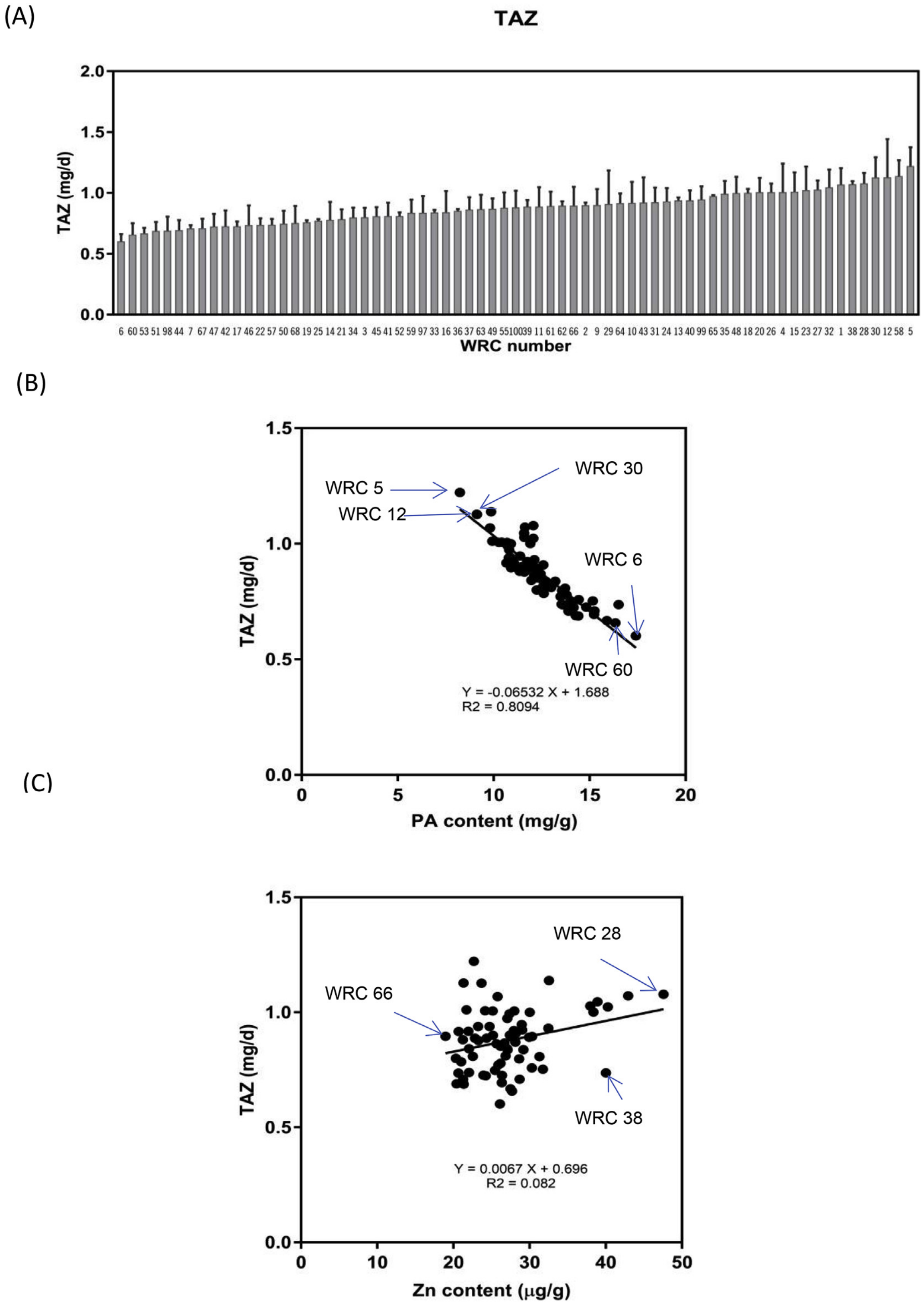


**Fig. 2.** Evaluation of the total daily absorption of Zn in the WRC (A) TAZ of WRC (B) Correlation of PA content and TAZ (C) Correlation of Zn content and TAZ.

Genebank Project, NARO (https://www.gene.affrc.go.jp). Plant height was measured during our field experiment, whereas seed length, seed width and seed weight were measured from harvested seeds. PA and Zn content data were measured as described above. Correlations among the phenotypic traits of WRC were analyzed to identify other traits affecting the PA content.

### 2.6. Evaluation of low and high PA WRC accessions

Three low and three high PA WRC accessions were identified and selected for further analysis. The selected low PA content accessions; WRC 5, WRC 12 and WRC 30 have been originated in India, China and Nepal respectively and the high PA content accessions; WRC 6 was originated in Indonesia while WRC 22 and WRC 44 in Philippines (Kojima et al., 2005). Further, considering the geographical information of the accessions there was no any relation with the PA contents.

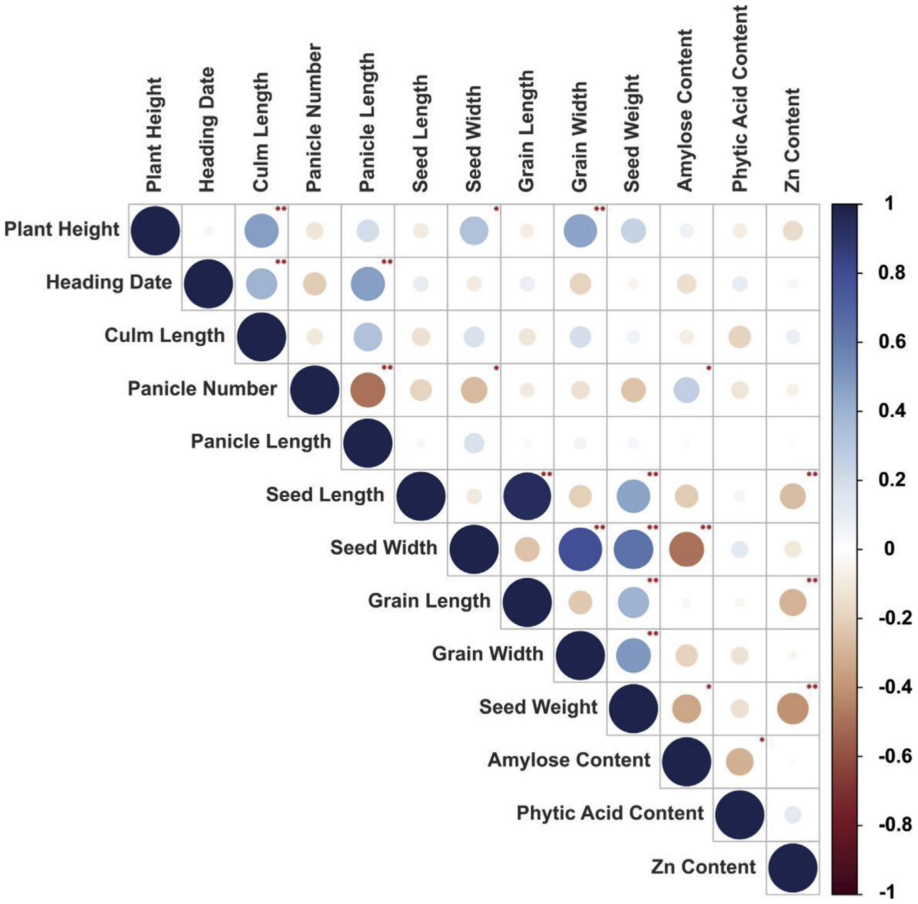


**Fig. 3.** Correlation matrix among phenotypic traits of 69 WRC. * and ** indicate significant differences at $p < 0.05$ and 0.01, respectively.

We also considered the number of days to heading and the panicle setting ability at the study location when selecting the accessions. Thus, three WRC accessions (WRC 60, WRC 53 and WRC 67) that had higher PA contents than WRC 44 (Fig. 1A) were not used in further experiments because WRC 60 and WRC 67 did not set panicles in the field conditions at Itakura, Gunma. Similarly, WRC 53 requires extra-long days for heading (as reported in 2016 from field observation data) and was not further analyzed. The content of other minerals and heavy metals of the low and high PA WRC accessions were analyzed as described above.

### 2.7. Statistical analysis

The statistics software Statistical Package for Social Sciences (SPSS), version 25.0 was used to analyze the experimental data. The results were expressed as mean ± standard deviation (SD). Comparison of differences between groups was analyzed by a one-way analysis of variance (ANOVA), and significant differences between group means were determined by Duncan's test at $p < 0.05$. Linear regression analysis was performed to observe the relationship among PA, Zn and TAZ. The Pearson correlation coefficient among phenotypic traits was calculated by the R statistical package, version 3.5.0.

### 2.8. Genome-wide association mapping

Genotyping data were obtained from a high-density rice array (HDRA) single-nucleotide polymorphisms (SNPs) database (http://www.ricediversity.org.) that consists of 700,000 SNPs (McCouch et al., 2016). Out of 69 WRC accessions, 62 accessions for which genotypic data is available were used for the analysis. To estimate the possible impact of population stratification, the population structure of the traits was tested before the association analysis. Genetic differences based on the pairwise identity-by-state (IBS) distances and a multi-dimensional scaling (MDS) analysis was implemented in PLINK (Purcell et al., 2007) to identify population structure. Association mapping was conducted using linear regression model with population stratification MDS dimensions as covariates to avoid false positives. SNP loci with more than a 10% missing rate and minor allele frequencies (MAF) of less than 0.05 were removed. After quality control, a total of 185,146 SNPs were used in the association study in PLINK software (Purcell et al., 2007) and a Manhattan plot was developed by Haploview 4.2 (Barrett et al., 2005) The SNPs within 1 Mbp were considered as the same locus and SNP sites with the lowest $P$ value in the peak region ($p < 10^{-4}$) were considered as significantly associated.

## 3. Results

### 3.1. Variation in the PA and Zn contents of WRC accessions

Variation in the PA and Zn contents and correlations between the PA and Zn contents of 69 WRC accessions are presented in Fig. 1. We have observed a range of 8.24–17.41 mg/g of PA content ($p < 0.05$ by one-way ANOVA) among the WRC accessions in our study. Three WRC accessions with the lowest PA content, WRC 5, WRC 12 and WRC 30 had PA contents of 8.24, 9.12 and 9.13 mg/g, respectively. The selected high PA WRC accessions, WRC 6, WRC 22 and WRC 44 had PA contents of 17.41, 16.51 and 16.34 mg/g, respectively (Fig. 1A). Relatively higher levels of variation in the Zn content were observed among the accessions, ranging from 18.95 to 47.56 μg/g. The highest Zn content was observed in WRC 28, whereas the lowest value was in WRC 66 (Fig. 1B). Furthermore, we did not observe a significant correlation ($p > 0.05$) between the PA and Zn contents among WRC accessions

(Fig. 1C).

### 3.2. Identification of high Zn bioavailable rice from the WRC

The calculated mean Zn intake from the WRC was from 0.602 to 1.222 mg Zn/day. The highest TAZ value was observed in WRC 5 that had the lowest PA content. The lowest Zn absorption was observed in the WRC 6 that had the highest PA content (Fig. 2A). Moreover, WRC 28 and WRC 38 that had elevated Zn contents had high TAZ values, whereas WRC 66 had a moderate TAZ value (Fig. 2A). A negative correlation ($p < 0.05$) was observed between the PA content and TAZ values (Fig. 2B), whereas the correlation was positive ($p < 0.05$) between the Zn content and TAZ values (Fig. 2C).

### 3.3. Correlation among morphological traits

The correlation coefficients among phenotypic traits of WRC accessions are presented in Fig. 3. The PA content was negatively correlated ($p < 0.05$) with the amylose content, and no significant correlations were observed with any other phenotypic traits. The Zn content was significantly ($p < 0.05$) and negatively correlated with seed length, grain length and seed weight.

### 3.4. Variation in the mineral and heavy metals contents

Mineral and heavy metal contents of low and high PA rice accessions are shown in Table 1. There were no significant differences ($p > 0.05$) in the contents of Ca, Mn, Fe and Cu among the three low and three high PA accessions. Levels of Ca, Mn, Fe and Cu in low PA rice ranged from 105.0 to 115.7 μg/g, 18.2–29.1 μg/g, 7.9–9.5 μg/g and 2.5–3.9 μg/g, whereas the high PA accessions ranged from 109.4 to 126.3 μg/g, 24.5–38.8 μg/g, 10.2–12.9 μg/g and 2.9–3.9 μg/g, respectively. Among the accessions, WRC 30 and WRC 5 had the lowest Ca and Mn contents respectively, whereas WRC 22 had the highest levels of these two minerals. The Fe content in high PA accessions were higher than those of low PA accessions, though not significant.

The heavy metal content varied randomly among the WRC accessions (Table 1). No substantial differences were noted for the heavy metal content between low and high PA groups; however, WRC 30 accumulated a significantly higher level of Cd among the accessions. There was no significant difference in Hg accumulation among the WRC accessions.

### 3.5. Genome-wide association study

GWA mapping on PA content identified 12 significant loci located on Chromosomes 1, 2, 3, 5, 6, 7, 8,11 and 12 (Fig. 4A, Table 2). Among these identified SNPs, no gene coding regions co-located with PA biosynthetic or accumulation-related genes. Further, 17 and 7 significant loci identified through GWA analysis on Zn Content (Supplementary Fig. S1) and TAZ (Supplementary Fig. S2) respectively. SNP positions determining PA content do not co-localize with SNPs for Zn.

## 4. Discussion

Exploring the genetic variation present in a germplasm collection is one of the major and preliminary steps for improving the desired trait in a breeding program. High levels of variation for PA and Zn contents were found in WRC accessions in this study, probably due to the broad geographic and genotypic variation existing in the collection (Kojima et al., 2005). Earlier studies also observed significant variation for the PA content and Zn content in rice (Welch and Graham, 2004). The high grain Zn genotypes will be an important genetic resource for breeding Zn-rich varieties.

The amount of Zn in the diet alone affects Zn absorption. In our study, we found two WRC accessions that had high Zn contents of 47.6 μg/g and 42.9 μg/g in brown rice (Fig. 1B); however, to benefit from the increased nutritive value of the grains, the bioavailability of the nutrient is essential (Trijatmiko et al., 2016). Based on a reference value for the daily consumption of rice by an adult human as 400 g, the average daily intake of PA is quite high, and the total daily level of dietary Zn is low. Thus, Zn bioavailability would be relatively low due to the combination of high PA content and moderately low Zn content in rice grain. Similar results were found for *lpa* maize, rice and barley using a suckling rat pup model that reported an inverse relationship between grain-derived dietary PA and Zn absorption (Lönnerdal et al., 2011). Based on the PA and Zn contents of rice and the values for TAZ, it is evident from our study that the low PA trait rather than high Zn content is more beneficial for increasing Zn bioavailability.

There was no obvious difference among the low and high PA rice accessions for Ca, Mn, Fe or Cu contents (Table 1). Significant increases in Ca (+20%) and Fe (+16%) were reported in the *indica*-type *lpa* rice mutant *Os-lpa-XQZ-1* (Frank et al., 2009). Notably, there was not an obvious correlation between the PA content and mineral content in two *japonica*-type mutants, *Os-lpa-XS110-1* and *Os-lpa-XS-110-2,* compared to the corresponding wild-type. Studies on an Azucena x IR64 population showed significant positive correlations between phytate and Fe, Zn, Cu and Mn levels; however, the QTLs for phytate and these minerals were not located within the same chromosomal regions and were genetically different (Stangoulis et al., 2007). Sakai et al. (2015) reported that the mineral content of *lpa* seeds was identical to the corresponding wild-type and that the PA content did not affect the translocation of

**Table 1**

Phytic acid and other mineral content of identified low and high PA rice accessions. Data are shown as mean ± standard deviation from three replicates[a].

| | Low PA | | | | | | High PA | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | WRC 05 | | WRC 12 | | WRC 30 | | WRC 06 | | WRC 22 | | WRC 44 | |
| *PA (mg/g)* | *8.2 ± 1.6* | a | 9.1 ± 2.8 | a | 9.1 ± 1.6 | a | 17.4 ± 1.6 | b | 16.5 ± 1.7 | b | 15.2 ± 2.8 | b |
| *Mineral (μg/g)* | | | | | | | | | | | | |
| Zn | 22.7 ± 2.5 | b | 21.3 ± 5.5 | b | 23.7 ± 3.1 | b | 26.1 ± 4.3 | b | 40.0 ± 6.2 | a | 26.4 ± 2.6 | b |
| Ca | 106.4 ± 6.1 | ab | 115.7 ± 5.9 | ab | 105.0 ± 3.6 | b | 125.7 ± 12.1 | a | 126.3 ± 10.4 | a | 109.4 ± 5.3 | ab |
| Mn | 18.2 ± 3.0 | c | 25.0 ± 6.2 | bc | 29.1 ± 8.0 | abc | 24.5 ± 4.9 | bc | 38.8 ± 9.9 | a | 35.7 ± 2.4 | ab |
| Fe | 9.5 ± 1.3 | b | 7.9 ± 1.0 | b | 9.0 ± 0.2 | b | 12.9 ± 2.7 | a | 10.2 ± 0.6 | ab | 10.3 ± 1.7 | ab |
| Cu | 3.0 ± 0.2 | bc | 2.5 ± 0.2 | c | 3.9 ± 0.3 | a | 2.9 ± 0.5 | bc | 3.9 ± 0.7 | a | 3.8 ± 0.7 | ab |
| *Heavy metal (μg/g)* | | | | | | | | | | | | |
| As | 0.093 ± 0.034 | c | 0.168 ± 0.036 | a | 0.146 ± 0.009 | ab | 0.108 ± 0.026 | bc | 0.133 ± 0.043 | abc | 0.147 ± 0.007 | ab |
| Cd | 0.016 ± 0.001 | b | 0.006 ± 0.000 | c | 0.051 ± 0.010 | a | 0.015 ± 0.001 | b | 0.011 ± 0.001 | bc | 0.010 ± 0.002 | bc |
| Sn | 0.006 ± 0.001 | b | 0.004 ± 0.000 | d | 0.006 ± 0.000 | b | 0.005 ± 0.000 | bc | 0.004 ± 0.000 | cd | 0.007 ± 0.000 | a |
| Hg | 0.036 ± 0.001 | a | 0.037 ± 0.004 | a | 0.037 ± 0.002 | a | 0.041 ± 0.001 | a | 0.041 ± 0.008 | a | 0.039 ± 0.004 | a |
| Pb | 0.021 ± 0.005 | bc | 0.017 ± 0.001 | c | 0.034 ± 0.007 | ab | 0.024 ± 0.008 | bc | 0.020 ± 0.004 | c | 0.040 ± 0.006 | a |

[a] Means within each row followed by the same letter are not significantly different at $p < 0.05$.

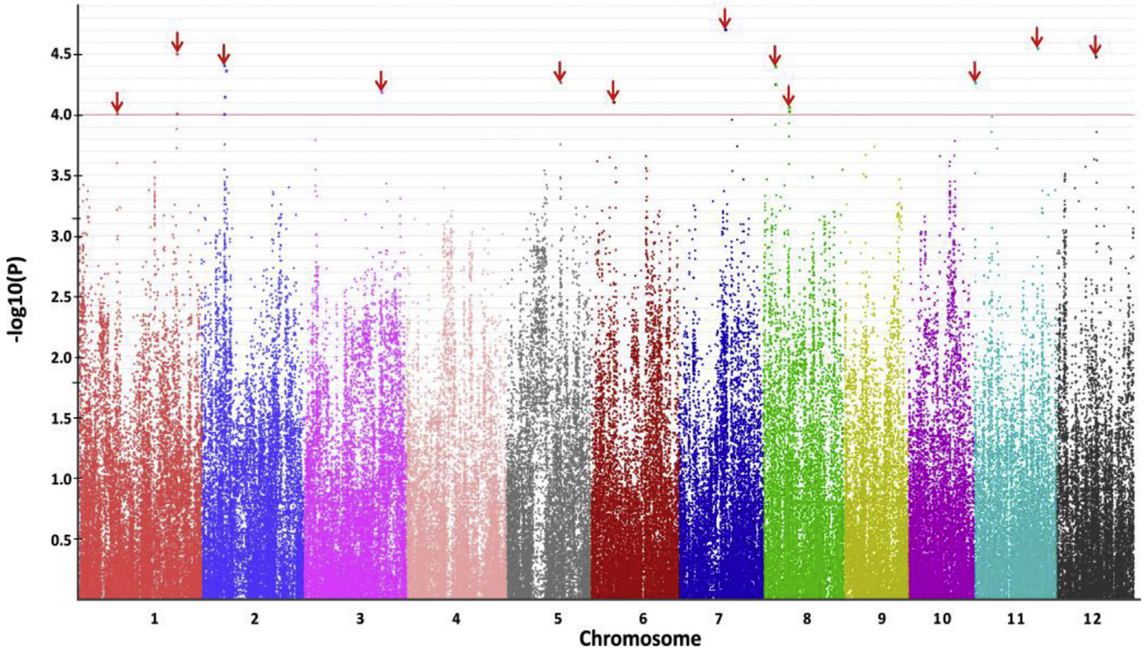


**Fig. 4.** Manhattan plot of the genome-wide association study for - PA content. The red line shows the threshold level ($p < 1 \times 10^{-4}$). Negative $\log_{10}$ transformed $p$ values are plotted against the position on each of the 12 chromosomes. The significant SNPs are marked with downward arrows.

**Table 2**
Significant SNPs for PA content in WRC accessions identified by GWAS.

| Chromosome | SNP identifier | Physical Position (bp) | *P* value |
|---|---|---|---|
| 1 | SNP-1.13341885. | 13342912 | 9.65E-05 |
| 1 | SNP-1.34444598. | 34445642 | 3.11E-05 |
| 2 | SNP-2.8009451. | 8009453 | 3.87E-05 |
| 3 | SNP-3.27727809. | 27734757 | 6.41E-05 |
| 5 | SNP-5.18978946. | 19041463 | 5.32E-05 |
| 6 | SNP-6.8016079. | 8017079 | 7.76E-05 |
| 7 | SNP-7.16299054. | 16300048 | 1.96E-05 |
| 8 | SNP-8.4390768. | 4391766 | 3.94E-05 |
| 8 | SNP-8.9166126. | 9167123 | 8.53E-05 |
| 11 | SNP-11.373613. | 374612 | 5.38E-05 |
| 11 | SNP-11.21966709. | 22432839 | 2.79E-05 |
| 12 | SNP-12.13915993. | 13918656 | 3.26E-05 |

mineral elements from vegetative organs into seeds but caused changes in the mineral localization in the seed. These results suggest that there might be different mechanisms controlling these traits, so that the PA content can be improved without affecting the acquisition and accumulation of mineral elements.

Analysis of the heavy metal content in low and high PA WRC accessions revealed considerable variation in concentration that differed among genotypes, except for Hg accumulation (Table 1). Previous studies on *Os-lpa-XS110-2* observed that its Cd content ranged from 19 to 158 mg/kg at different locations (Frank et al., 2009). To date, there are no reports on other heavy metal levels in *lpa* rice mutants that would help to understand any correlation or impact of reduced PA content on heavy metal concentration. In accordance with our results, there seemed to be no relation between the heavy metals and PA content in rice grain. Rather, the condition of the soil and the environment may affect the accumulation and distribution of the minerals and heavy metals in rice grain.

Correlation analysis among the phenotypic traits is useful for understanding their relationships. In our study, several interrelationships were observed among the traits, however, the PA content only had a significant negative correlation with the amylose content (Fig. 3). In contrast, the significant SNPs identified by GWA analysis for amylose content (Supplementary Fig. S3) did not overlap with significant SNPs associated with the PA content (Fig. 4). This result suggests that PA and amylose accumulation are independently regulated. PA content was also independent from Zn content (Fig. 3). In GWA analysis, we did not observe common significant SNP positions among PA and Zn (Fig. 4, Supplementary Fig. S1) which also evident that PA and Zn accumulation is regulated by independent genes. The observed negative correlation of Zn content and the seed weight of rice grain indicates a dilution effect in grain, whereas the lack of any significant correlation of PA with seed size traits implies that PA accumulation is independent from grain filling.

By GWA analysis, there were 2 common significant SNPs between PA and TAZ while no common SNPs between Zn and TAZ which highlights that TAZ genetically determined by PA trait. The significant SNPs identified in this study for PA, Zn and TAZ do not co-localize with the identified PA biosynthetic genes (Supplementary Fig. S4) (Perera et al., 2018). However, four SNPs are located on chromosome 2 where important PA biosynthetic genes (*IPK2*, *2-PGK*, *ITPK4* and *IMP-2*) are located. The significant SNP on chromosome 7 is also located close to the *MIK* gene, a key gene in PA biosynthesis and accumulation in rice. Chromosome 3 has five known PA accumulation genes; however, only one significant SNP was observed in our study. The locations of the other SNPs that are on Chromosomes 1, 5, 8, 11 and 12 have not previously been identified as having PA genes. These results indicate that the PA content is under genetic control, not only by mutation within the coding region of PA biosynthesis genes but also other mutations within unknown genes or *cis*-regulatory regions that may affect the expression of PA biosynthesis genes. Novel type of molecular mechanisms regulating grain PA contents might be harbored around found SNPs. Further, other mechanisms such as P uptake, translocation and remobilization inside the plant may have considerable effects on PA accumulation in seeds.

The results suggest that Zn bioavailability in rice is strongly affected by the PA content of the grain which highlights the fact that decreasing the concentration of PA in rice is a possible strategy for improving Zn bioavailability. Thus, reducing the PA content in rice seems an important target for reducing Zn deficiency in populations that consume rice as their main food component, especially in developing countries. The stability of PA and mineral elements that accumulate in these accessions should be evaluated over different environments to understand the genotypic and environmental effects. Further, additional investigation is needed to know more about the mechanism of PA accumulation in rice grain and to understand the genetic architecture of PA content.

## Abbreviations used

ALP, alkaline phosphatase; GWAS, genome-wide association study; ICPMS, inductively coupled plasma mass spectrometry; *lpa*, low phytic acid; PA, phytic acid; QTL, quantitative trait loci; SNP, single nucleotide polymorphism; TAZ, total daily absorption of Zn; WRC, World Rice Core collection.

## Funding

This work was supported by an Enryo Inoue Research Grant, Toyo University, Japan. We also thank the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan for the Monbukagakusho scholarship for IP.

## Conflicts of interest

The authors declare no competing financial interest.

## Author contributions

IP, SS and NH conceived the research project and designed the study. IP carried out all experiments, AF and KY performed the phytic acid analysis, MA and SN assisted with the elemental analyses. IP analyzed the datasets and GWA analysis. IP, SS and NH wrote the paper.

## Acknowledgements

The authors would like to acknowledge the NARO Genebank in the national germplasm collection in Tsukuba, Japan for providing the WRC seed materials for the study.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcs.2018.12.010.

## References

Al Hasan, S.M., Hassan, M., Saha, S., Islam, M., Billah, M., Islam, S., 2016. Dietary phytate intake inhibits the bioavailability of iron and calcium in the diets of pregnant women in rural Bangladesh: a cross-sectional study. BMC Nutr. 2, 24. https://doi.org/10.1186/s40795-016-0064-8.

Barrett, J.C., Fry, B., Maller, J., Daly, M.J., 2005. Haploview: Analysis and visualization of LD and haplotype maps. Bioinformatics 21, 263–265. https://doi.org/10.1093/bioinformatics/bth457.

Brown, K.H., Rivera, J.A., Bhutta, Z., Gibson, R.S., King, J.C., Lönnerdal, B., Ruel, M.T., Sandtröm, B., Wasantwisut, E., Hotz, C., 2004. International Zinc Nutrition Consultative Group (IZiNCG) technical document #1. Assessment of the risk of zinc deficiency in populations and options for its control. Food Nutr. Bull. 25, S91–S204. https://doi.org/10.1177/15648265040251S204.

FAO, 2013. FAO Statistical Yearbook 2013, Crop production statistics. Food and Agriculture Organization, Rome. https://doi.org/10.1017/CBO9781107415324.004.

Frank, T., Habernegg, R., Yuan, F.J., Shu, Q.Y., Engel, K.H., 2009. Assessment of the contents of phytic acid and divalent cations in low phytic acid (*lpa*) mutants of rice and soybean. J. Food Compos. Anal. 22, 278–284. https://doi.org/10.1016/j.jfca.2008.11.022.

Hambidge, K.M., Miller, L.V., Westcott, J.E., Sheng, X., Krebs, N.F., 2010. Zinc bioavailability and homeostasis. Am. J. Clin. Nutr. 91, 1478–1483. https://doi.org/10.3945/ajcn.2010.28674I.

IFPRI, 2016. Global Nutrition Report 2016 From Promise to Impact Ending Malnutrition by 2030. Washington DC. . https://doi.org/10.2499/9780896295841.

Kojima, Y., Ebana, K., Fukuoka, S., Nagamine, T., Kawase, M., 2005. Development of an RFLP-based Rice Diversity Research Set of Germplasm. Breed Sci. 55, 431–440. https://doi.org/10.1270/jsbbs.55.431.

Lee, H.H., Bong, C.F.J., Loh, S.P., Sarbini, S.R., Yiu, P.H., 2014. Genotypic, grain morphological and locality variation in rice phytate content and phytase activity. Emir. J. Food Agric. 26, 844–852. https://doi.org/10.9755/ejfa.v26i10.18503.

Liu, Q.L., Xu, X.H., Ren, X.L., Fu, H.W., Wu, D.X., Shu, Q.Y., 2007. Generation and characterization of low phytic acid germplasm in rice (*Oryza sativa* L.). Theor. Appl. Genet. 114, 803. 14. https://doi.org/10.1007/s00122-006-0478-9.

Lönnerdal, B., Mendoza, C., Brown, K.H., Rutger, J.N., Raboy, V., 2011. Zinc Absorption from low phytic acid genotypes of maize (*Zea mays* L.), barley (*Hordeum vulgare* L.), and rice (*Oryza sativa* L.) assessed in a suckling rat pup model. J. Agric. Food Chem. 59, 4755–4762. https://doi.org/10.1021/jf1043663.

Lott, J.N.A., Ockenden, I., Raboy, V., Batten, G.D., 2000. Phytic acid and phosphorus in crop seeds and fruits: a global estimate. Seed Sci. Res. 10, 11–33. https://doi.org/10.1017/S0960258500000039.

McCouch, S.R., Wright, M.H., Tung, C.-W., Maron, L.G., Mcnally, K.L., Fitzgerald, M., Singh, N., Declerck, G., Agosto-Perez, F., Korniliev, P., Greenberg, A.J., Elizabeth, M., Naredo, B., Mae, S., Mercado, Q., Harrington, S.E., Shi, Y., Branchini, D.A., Kuser-Falcão, P.R., Leung, H., Ebana, K., Yano, M., Eizenga, G., Mcclung, A., Mezey, J., 2016. Open access resources for genome-wide association mapping in rice. Nat. Commun. 7. https://doi.org/10.1038/ncomms10532.

McKie, V.A., McCleary, B.V., 2016. A novel and rapid colorimetric method for measuring total phosphorus and phytic acid in foods and animal feeds. J. AOAC Int. 99, 738–743. https://doi.org/10.5740/jaoacint.16-0029.

Miller, B.D.D., Welch, R.M., 2013. Food system strategies for preventing micronutrient malnutrition. Food Pol. 42, 115–128. https://doi.org/10.1016/j.foodpol.2013.06.008.

Miller, L.V., Krebs, N.F., Hambidge, K.M., 2007. A mathematical model of zinc absorption in humans as a function of dietary zinc and phytate. J. Nutr. 137, 135–141.

Mottaleb, K.A., Mishra, A.K., 2016. Rice consumption and grain-type preference by household: A Bangladesh case. J. Agric. Appl. Econ. 48, 298–319. https://doi.org/10.1017/aae.2016.18.

Perera, I., Seneweera, S., Hirotsu, N., 2018. Manipulating the phytic acid content of rice grain toward improving micronutrient bioavailability. Rice 11, 4. https://doi.org/10.1186/s12284-018-0200-y.

Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M.A.R., Bender, D., Maller, J., Sklar, P., de Bakker, P.I.W., Daly, M.J., Sham, P.C., 2007. PLINK: A tool set for whole-genome association and population-based linkage analyses. Am. J. Hum. Genet. 81, 559–575. https://doi.org/10.1086/519795.

Raboy, V., 2001. Seeds for a better future: “Low phytate” grains help to overcome malnutrition and reduce pollution. Trends Plant Sci. 6, 458–462. https://doi.org/10.1016/S1360-1385(01)02104-5.

Sakai, H., Iwai, T., Matsubara, C., Usui, Y., Okamura, M., Yatou, O., Terada, Y., Aoki, N., Nishida, S., Yoshida, K.T., 2015. A decrease in phytic acid content substantially affects the distribution of mineral elements within rice seeds. Plant Sci. 238, 170–177. https://doi.org/10.1016/j.plantsci.2015.06.006.

Sato, Y., Takehisa, H., Kamatsuki, K., Minami, H., Namiki, N., Ikawa, H., Ohyanagi, H., Sugimoto, K., Antonio, B.A., Nagamura, Y., 2013. RiceXPro Version 3.0: Expanding the informatics resource for rice transcriptome. Nucleic Acids Res. 41, 1206–1213. https://doi.org/10.1093/nar/gks1125.

Spindel, J., Begum, H., Akdemir, D., Virk, P., Collard, B., Redoña, E., Atlin, G., Jannink, J.L., McCouch, S.R., 2015. Genomic selection and association mapping in rice (*Oryza sativa*): effect of trait genetic architecture, training population composition, marker number and statistical model on accuracy of rice genomic selection in elite, tropical rice breeding lines. PLoS Genet. 11, e1004982. https://doi.org/10.1371/journal.pgen.1004982.

Stangoulis, J.C.R., Huynh, B.L., Welch, R.M., Choi, E.Y., Graham, R.D., 2007. Quantitative trait loci for phytate in rice grain and their relationship with grain micronutrient content. Euphytica 154, 289–294. https://doi.org/10.1007/s10681-006-9211-7.

Trijatmiko, K.R., Duenãs, C., Tsakirpaloglou, N., Torrizo, L., Arines, F.M., Adeva, C., Balindong, J., Oliva, N., Sapasap, M.V., Borrero, J., Rey, J., Francisco, P., Nelson, A., Nakanishi, H., Lombi, E., Tako, E., Glahn, R.P., Stangoulis, J., Chadha-Mohanty, P., Johnson, A.A.T., Tohme, J., Barry, G., Slamet-Loedin, I.H., 2016. Biofortified indica rice attains iron and zinc nutrition dietary targets in the field. Sci. Rep. 6, 1–13. https://doi.org/10.1038/srep19792.

Wang, K.M., Wu, J.G., Li, G., Zhang, D.P., Yang, Z.W., Shi, C.H., 2011. Distribution of phytic acid and mineral elements in three indica rice (*Oryza sativa* L.) cultivars. J. Cereal. Sci. 54, 116–121. https://doi.org/10.1016/j.jcs.2011.03.002.

Welch, R.M., Graham, R.D., 2004. Breeding for micronutrients in staple food crops from a human nutrition perspective. J. Exp. Bot. 55, 353–364. https://doi.org/10.1093/jxb/erh064.

WHO, 2002. The world health report 2002 - reducing risks, promoting healthy life. Educ. Health 16, 230. https://doi.org/10.1080/1357628031000116808.

Xu, F., Bao, J., Kim, T.S., Park, Y.J., 2016. Genome-wide association mapping of polyphenol contents and antioxidant capacity in whole-grain rice. J. Agric. Food Chem. 64. https://doi.org/10.1021/acs.jafc.6b01289.

Zhao, K., Tung, C.-W., Eizenga, G.C., Wright, M.H., Ali, M.L., Price, A.H., Norton, G.J., Islam, M.R., Reynolds, A., Mezey, J., McClung, A.M., Bustamante, C.D., McCouch, S.R., 2011. Genome-wide association mapping reveals a rich genetic architecture of complex traits in *Oryza sativa*. Nat. Commun. 2, 467. https://doi.org/10.1038/ncomms1467.